STUDIES OF NITROGEN FIXING BACTERIA. IX

Study of Inoculation of Wheat with Azotobacter in Laboratory and Field Experiments

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(Plates xxvii–xxviii)

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Synopsis

Azotobacter used as inoculum on wheat seed can multiply during the germination of the seed. The organic substances exuded by the seed provide the necessary carbon source to support the growth of *Azotobacter*. However, multiplication was subject to competition from other micro-organisms present on the seed coat and in the soil. On agar media or in sand culture, *Azotobacter* was capable of multiplying in the presence of other micro-organisms if combined nitrogen were not added into the media. In soil, the numbers of *Azotobacter* increased during the early germination of wheat seeds but were reduced at later stages.

In field trials our experimental results do not confirm claims that *Azotobacter* inoculation increases crop yields.

Introduction

Agricultural experiments with *Azotobacter* have been carried out since 1902 (Gerlach and Vogel, 1902). Since this date, a considerable amount of data has accumulated; review papers by Cooper (1959) and Rubenchik (1960) have summarized the earlier work. More recently several papers have been published dealing with the use of *Azotobacter* as an inoculum and the development of the organism in the rhizosphere (Brown *et al.*, 1962; Helmeczi, 1963; Katznelson and Strzelczyk, 1961; Macura, 1963; Maliozewska, 1961; Němec and Pecina, 1964; Pochon, 1963; Panosyan, 1964; Rakhno and Rȳȳs, 1963; Rovira, 1963; Samtsevich, 1963; Starkey, 1961; Strzelczyk, 1961; Vancura, 1964).

The literature gives the impression that seed inoculation by non-symbiotic micro-organisms is inconsistent in its effects on plant yields. No logical general explanation has yet been proposed.

In view of the possible importance of *Azotobacter* inoculation to yield increase in the wheat industry of Australia, and since no such experiments on New South Wales wheat soils were available, the authors have examined the effects of *Azotobacter* on the growth of wheat under laboratory and field conditions.

Methods

The effects of *Azotobacter* were examined in laboratory trials using sterile media, sand and soil cultures, and unsterilized cores of soil, and in a number of field trials. Wheat, *Triticum aestivum* L. var. 'Gabo' was used in the laboratory trials; var. 'Festival' was used in the field trials. However, the wheat used in some earlier experimental work done in France was of unknown variety.

(*a*) Laboratory trials

Organisms used: *Azotobacter chroococcum* strains NG241, Veg. B, Veg. 2, Sydney peggy, wheat root (a strain of *Azot. chroococcum* isolated from wheat rhizosphere), IP1, IP2; *Azotobacter macrocytogenes*; *Azotobacter vinelandii* (obtained from Delft, Holland); *Azomonas agilis*; *Azomonas insigne*.

Media

(A) Winogradsky nitrogen free mineral medium: K_2HPO_4, 1 g; NaCl, 0·5 g; $MnSO_4,4H_2O$, 0·01 g; $MgSO_4,7H_2O$, 0·5 g; $FeSO_4,7H_2O$, 0·01 g; tap water, 1,000 ml.

(B) Ammonium nitrate: 1% solution.

(C) Winogradsky mineral agar medium: Solution (A) with 1·5% agar.

(D) Winogradsky sucrose agar medium: Medium (C) with 1% sucrose.

(E) Sand: Coarse river sand, washed with tap water.

(F) Soil cores were collected in tins driven into the soil to a depth of four inches. Samples were taken from a long-cultivated wheat field at Tamworth, N.S.W., (approx. 34 crops) and from an adjacent area that had never been cropped.

Seed sterilization techniques: Seeds were washed in detergent (5% teepol), then in 1% calcium hypochlorite for 10 minutes. The hypochlorite was removed by a series of 10 rinses in sterile distilled water.

Azotobacter counting technique: The number of *Azotobacter* was determined by the method described previously by Tchan (1952).

(*b*) Field experiments

During the 1962 season, field trials of *Azotobacter* inoculation of wheat were carried out in the vicinity of Tamworth, N.S.W.

A 2 × 3 × 2 factorial design was used to examine the effects of *Azotobacter* and the interactions due to added phosphorus and nitrogen fertilizers.

Table 1

Chemical analyses of soil samples from the land sown to the factorial trial and from adjacent uncultivated land

	Cultivated Soil (Trial Area)		Adjacent Uncultivated Soil	
	0—4″	4″—8″	0—4″	4″—8″
pH (" sticky point " method)	6·0	6·5	6·0	6·3
Extractable phosphorus				
Truog (p.p.m.) ..	0	0	0·4	0
Olsen (p.p.m.)	0·4	2·6	10·9	1·9
Bray (p.p.m.)	3·7	1·4	4·5	2·1
Organic Carbon				
Tinsley (%)	0·99	0·78	1·69	1·16

The trial was carried out on a solodized Red-Brown Earth soil (Dr2·23—Northcote, 1960) that had been under cultivation for about 20 years, with a rotation based on several years of grazing lucerne (*Medicago sativa* L.) followed by some years of wheat cropping. 1962 was the first year of cropping following a period under lucerne. Table 1 shows a comparison of chemical analyses of the cultivated soil and of samples taken from an adjacent area that had been cleared and grazed, but had never been cultivated.

The decline in organic carbon and extractable phosphorus due to cultivation is typical of the differences found in similar comparisons in this district.

The factors and levels used in the trial were: (1) *Azotobacter* inoculation: not inoculated, inoculated; (2) Superphosphate: 0, 80 and 160 lb/acre; (3) Sulphate of ammonia: 0, 50 lb/acre.

The treatments were fully randomized in each of two replicates.

The inoculation treatment was applied by steeping 5 lb. of seed in 40 oz. of water containing 20 ml. of a 7-days-old *Azotobacter* (strain IP_1) suspension (10^9 cells/ml.) for 30 minutes. After soaking, the grain was spread out in a shaded place. The uninoculated seed was steeped in the same way, in water without the *Azotobacter* suspension.

The seed was sown from a commercial cultivator-drill within a couple of hours from inoculation or soaking. The seed and fertilizer were separate up to the time of sowing, but were brought into contact in the delivery tube of the drill during sowing and were sown together in the drill row in the soil. The sowing rate was 47 lb/acre.

The plots were nine rows wide (4′8″) by 130 ft. long. At harvest the length was trimmed to 124′6″ and the middle five rows were harvested to give the yield from 1/120 acre.

In addition, comparison trials were sown at nine other sites in the district, these consisted of uninoculated and inoculated plots, and were designed to measure the responses to inoculation over a range of farm soils. From these nine and the factorial trial ten sets of data were available for comparison.

Results and Discussion

(1) Multiplication of *Azotobacter* in the presence of wheat seedlings in agar medium.

Wheat seeds (of an unknown French variety) were sterilized as previously described. This simplified the interpretation of the results by eliminating the interaction of the microflora of the wheat rhizosphere. The sterile seeds were inoculated with one drop of a suspension of a culture of *Azotobacter* strain IP_1 (agar culture) and transferred to Winogradsky's mineral agar medium. The seeds were then allowed to germinate at room temperature. After one week the wheat roots were found to be covered with *Azotobacter* (Plate xxvii, *a*, *b*, *c*). This was reisolated and found to be identical with the original inoculum. The experiment was repeated and the results were reproduced. This result indicated that the culture of *Azotobacter* used was capable of multiplication in the rhizosphere of a wheat seedling without added organic substances.

After these preliminary encouraging results, other strains of *Azotobacter chroococcum* (including IP_1) and other species of *Azotobacter* and *Azomonas* as listed above (under Methods, p. 290) were tested in Australia in a similar manner with local wheat varieties. The results were all negative. There was no growth of *Azotobacter* or *Azomonas* in the rhizosphere of the wheat seedling including the strain of *Azotobacter chroococcum* (wheat-root) originally isolated from a wheat rhizosphere.

In spite of the reproducible results of the early experiment it was impossible to repeat the colonization of the rhizosphere of wheat by *Azotobacter*. Close examination of the plates showed that the sites where the seeds were deposited, before their displacement due to germination, contained numerous small colonies of *Azotobacter* and *Azomonas* (Plate xxviii). This suggested that during germination, at least under these artificial conditions, enough organic substances had been exuded to support limited growth of *Azotobacter in situ*. Also, it appears that if the seeds are inoculated, under such conditions, an increase in *Azotobacter* surrounding the seedling at the early stage of germination may be expected without establishing a true rhizosphere association. Such growth could provide some growth factors or plant hormone-like substances (known to be excreted by *Azotobacter*) to influence the growth of wheat. Therefore, it was decided to examine the effects of *Azotobacter* on wheat grown to a more advanced stage in sand culture and in soil.

(2) Multiplication of *Azotobacter* in contact with wheat seeds in plant tube cultures.

To obtain more quantitative information a sand culture technique was used. 10 ml. of Winogradsky mineral solution was added to 50 g. of air dried sand in 3·5 cm. diameter tubes. The tubes were then sterilized at 15 lb. for 20 minutes.

The tubes were inoculated with 5 ml. of suspension of *Azotobacter chroococcum* strain IP_1 containing 500 cells per ml. (a total of 2,500 cells). Immediately after inoculation, 35 ml. of mineral medium was added to the control tube (total volume of 50 ml.) and ten-fold dilutions carried out to estimate the initial number. The other tubes were seeded with six wheat grains either killed (by boiling the seed in water), surface sterilized, or unsterile. In the nitrogen treatment 0·15 ml. of 1% ammonium nitrate was added to the tubes.

At seven and 21 days, the number of *Azotobacter* per tube was estimated and the plant growth was measured by length in cm. The results are summarized in Table 2.

In the absence of added nitrogen there was an increase in *Azotobacter* in all cases. The killed seeds, the surface sterilized and unsterilized seeds all provided an organic exudate for the *Azotobacter* multiplication. Where nitrogen was added, multiplication again occurred in the presence of the killed and surface sterilized seed but not in the case of unsterilized seed.

The multiplication of *Azotobacter* did not influence the growth of seedlings. At seven days level the interpretation is difficult since the length of the seedlings is influenced by the initial germination energy. For all practical purposes, the difference cannot be regarded as having any importance.

The above experiment indicated that the utilization of the organic matter from exudates by *Azotobacter* in the case of unsterile seed is subject to competition from micro-organisms carried by the seed coat.

(3) Multiplication of *Azotobacter* in soil in the presence of wheat seedlings

In soil, the micro-flora could also influence the multiplication and survival of *Azotobacter* in seed inoculation experimentation. Also the amount of available mineral nitrogen can rarely reach 0·01% (100 p.p.m.). Therefore the competition may not be as severe as in the sand experiment with added NH_4No_3

The experiment was repeated using soil from a wheat field in place of sand. Calcium carbonate was added to produce a near neutral pH. The soil was not sterilized and the ammonium nitrate treatment was omitted. The results are included in Table 2.

The seven day count showed an initial increase in *Azotobacter*, but at 21 days the numbers had fallen to less than the original inoculation.

These results suggest that limited growth of *Azotobacter* is possible during the early stages of germination.

(4) Effects of *Azotobacter* in pot trials

Soil cores were collected from cultivated and uncropped soil at Tamworth, N.S.W. Calcium carbonate was added to the surface to bring the pH approximately to neutrality.

A 2^3 *Azotobacter* × phosphorus × nitrogen trial was made. The preliminary result indicated that wheat seed without fertilizer responded very slightly but not significantly to *Azotobacter* inoculation. With fertilizer treatment, there was no response to inoculation. The detailed results are not reported here.

TABLE 2

Multiplication of Azotobacter *in the presence of wheat seeds* (initial number of Azotobacter introduced as inoculum $0 \cdot 25 \cdot 10^4$)

	Wheat Grains		
	Killed	Sterilized	Unsterilized
SAND CULTURE NH_4NO_3 not added			
7 days incubation			
No. of Azotobacter per tube ..	$40 \cdot 4 \cdot 10^4$	$41 \cdot 10^4$	$16 \cdot 7 \cdot 10^4$
Mean value of seedling length			
inoculated ..	—	13 cm.	16
uninoculated* ..	—	6	11·6
21 days incubation			
No. of Azotobacter per tube ..	$31 \cdot 3 \cdot 10^4$	$7 \cdot 10^4$	$4 \cdot 7 \cdot 10^4$
Mean value of seedling length			
inoculated ..	—	21·6	21·5
uninoculated* ..	—	20·7	23·6
	NH_4NO_3 added (final concentration 0·01%)		
7 days incubation			
No. of Azotobacter per tube ..	$20 \cdot 10^4$	$7 \cdot 10^4$	$0 \cdot 1 \cdot 10^4$
Mean value of seedling length			
inoculated ..	—	11·9	12
uninoculated* ..	—	6	10·6
21 days incubation			
No. of Azotobacter per tube ..	$37 \cdot 10^4$	$5 \cdot 10^4$	$0 \cdot 6 \cdot 10^4$
Mean value of seedling length			
inoculated ..	—	26	26
uninoculated* ..	—	26	27
SOIL CULTURE (unsterilized)			
7 days incubation			
No. of Azotobacter per tube ..	$2 \cdot 7 \cdot 10^4$	$17 \cdot 10^4$	$2 \cdot 2 \cdot 10^4$
Mean value of seedling length			
inoculated ..	—	12	13
uninoculated ..	—	9	13
21 days incubation			
No. of Azotobacter per tube ..	$0 \cdot 2 \cdot 10^4$	0	$0 \cdot 002 \cdot 10^4$
Mean value of seedling length			
inoculated ..	—	19	27
uninoculated ..	—	23	25

* No Azotobacter was detected at any stage of seedling growth.

A 2 × 2 trial with eight replications was set out to ascertain the effect of inoculation in cultivated and uncultivated soil. The wheat seeds for the inoculation treatment were soaked in a suspension of *Azotobacter* strain IP_1 for one hour. Seeds for the uninoculated treatment were soaked in water for the same time. The soil cores were manipulated with a minimum disturbance possible to the soil structure. Each pot received six seeds and was later thinned to four seedlings which were allowed to grow for 74 days in glasshouse conditions. The pot arrangement and the results were as indicated in Table 3.

Statistical analysis showed that there were no significant differences of plant weight between the inoculated and the uninoculated treatments.

TABLE 3

Inoculation experiment in cultivated and uncultivated wheat soil. (Dry weight in g. of 4 wheat plants)

	Uncultivated soil				Total	Mean	Cultivated soil				Total	Mean
Inoculated I	1·07	0·94	0·88	0·60	7·19	0·898	0·56	0·56	0·87	0·88	5·84	0·73
Inoculated II	0·870	1·02	0·97	0·84			0·71	0·70	0·78	0·78		
Uninoculated I	0·7	0·95	0·87	0·75	6·34	0·906	0·62	0·78	1·06	0·71	4·80	0·685
Uninoculated II	0·90	0·83	1·34	—			0·56	0·62	0·45	—		

(5) Study of *Azotobacter* inoculation in field trials

To complete the investigation, field trials as described above (under Methods, p. 291) were sown in May, 1962. Inspections of the plots during the growing season and visual scoring for growth revealed no response to the *Azotobacter* inoculation.

At harvest the plots were well grown and free from weeds. There was no damage due to hail, disease, frost or lodging. A summary of the grain yields is presented in Table 4. The accuracy of the results is indicated by the low coefficient of variation (4·9%).

TABLE 4

Field Factorial trial. Grain yields in bushels per acre. (Means of two replicates)

Superphosphate lb/acre	0		80		160		Means
Sulphate of Ammonia lb/acre	0	50	0	50	0	50	
Azotobacter							
Not inoculated ..	19·7	22·3	26·6	27·6	30·0	29·3	25·9
Inoculated.. ..	21·4	19·3	27·8	26·5	29·9	29·8	25·8
Differences due to Azotobacter inoculation	+1·7	—3·0	+1·2	—1·1	—0·1	+0·5	—0·1

In the absence of fertilizers the difference in yield due to inoculation was an increase of 1·7 bushels per acre (on means of two plots). Over the whole trial, however, the mean effect of inoculation was a yield reduction of 0·1 bushels per acre. Neither of these results was statistically significant.

The results indicated that there was an *Azotobacter* × phosphorus × nitrogen interaction. Nitrogen appeared to be the dominant factor in the

interaction. In the absence of nitrogen, the responses to inoculation at 0, 80 and 160 lb/acre of superphosphate were + 1·7, + 1·2 and — 0·1 bushels per acre. In the presence of added nitrogen the corresponding responses were — 3·0, — 1·1 and + 0·5 bushels per acre. None of the interactions were statistically significant.

The experimental data with sand and soil under laboratory conditions (Table 2) indicated that the multiplication of *Azotobacter* in the presence of unsterilized seed was influenced by the available nitrogen. After a week in sand cultures the number of *Azotobacter* had dropped below the inoculum level where the available nitrogen was high. In the absence of added N, the number of *Azotobacter* had significantly increased. The situation was not very different after three weeks. The number of *Azotobacter* was still substantially higher in the no nitrogen treatment.

In the soil under laboratory conditions, with no fertilizer added, the number of *Azotobacter* increased only ten-fold during the first week and dropped well below the inoculated number in three weeks. It would not be unreasonable to speculate that in the presence of nitrogenous fertilizer *Azotobacter* inoculated with the seed would not increase but probably decrease and it could not exercise any influence on the wheat growth. The negative response at 50 lb/acre of sulphate of ammonia of —3·0 (no superphosphate) and of —1·05 (at 80 lb/acre of superphosphate) in the inoculated trial can not be explained on a microbiological basis.

Comparison plots

Inoculated and uninoculated plots were sown at nine other sites in the vicinity of Tamworth, N.S.W. Together with data from the factorial trial these gave ten sets of data. Two of the sites were on Black Earth soils; the others were on Solodized Red-Brown Earths. The data are summarized in Table 5.

Table 5

Grain yield from comparison plots. Bus/ac.

Soil Group	Inoculation treatment		Response
	Uninoculated	Inoculated	
Red-Brown Earth	19·7	21·4	+1·7
	14·0	12·1	—1·9
	8·5	7·8	—0·7
	27·9	25·8	—2·1
	8·6	8·0	—0·6
	19·6	20·7	+1·1
	15·8	23·4	+7·6
	14·0	14·6	+0·6
Black Earth	41·1	34·1	—7·0
	17·8	17·7	—0·1
Total	187·0	185·6	—1·4
Mean			—0·14

The mean response, — 0·14 bushels per acre, shows no benefit from inoculation. In two cases, however, the responses were marked. In the first, there was an apparent response in favour of inoculation (+ 7·6 bushels per acre); an inspection of the harvest records suggests that soil variation was the main reason for this apparent response. In the second, the difference was against inoculation (— 7·0 bushels per acre); no explanation can be given.

From the above data no apparent difference existed between the inoculated and uninoculated treatments.

Conclusions

It has been suggested that plant hormone-like substances or growth factors excreted by *Azotobacter* could be beneficial to the higher plant (see Starkey, 1961, and Pochon, 1963). Under laboratory conditions *Azotobacter* is capable of multiplication by utilizing the organic matter excreted during the germination of the seed. Therefore, such beneficial influence of *Azotobacter* should be noticeable in the early phase of plant growth. Our experiment in the laboratory and in the field failed to show such response by wheat seedling. Also, under our experimental conditions, the multiplication of *Azotobacter* during germination of the seed occurs only when the competition of other micro-organisms was not severe. When the available combined nitrogen is high *Azotobacter* can not compete successfully for the available organic matter. In the pot trials and field experiments, no significant beneficial effect could be obtained. Such conclusion may only apply to our experimental data ; however, when favourable ecological conditions are prevalent it could not be excluded that a possible significant influence of yields could be obtained by *Azotobacter* inoculation. To determine the most suitable ecological factors in this regard a very elaborate programme is needed. This would include the study of soil factors, climatic conditions, the investigation of interrelationship of micro-organism and higher plant, and genetic studies of biotic partners.

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EXPLANATION OF PLATES XXVII–XXVIII.

Plate xxvii.

Ia. Wheat seed germinated on agar medium. Note the colonies of *Azotobacter* surrounding the roots (arrows).

Ib. Detail of a root and root-hairs. Note the growth of *Azotobacter* in the rhizosphere (dark areas).

Ic. Detail of root tip and root-hairs with *Azotobacter*.

Plate xxviii.

II. Wheat seed germinated on agar medium. Micro-colonies of *Azotobacter* (arrows) using the exudate of the seed as organic matter. Note the absence of *Azotobacter* in the rhizosphere.